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13. Abstract *(Maximum 200 Words)* *(abstract should contain no proprietary or confidential information)*

The research goal is to demonstrate HDAC1 is a new chemotherapeutic target for human breast tumor cells and to identify new HDAC1 inhibitors on the basis of the structure of quinoline antimalarials. The drug screening strategy we used included 8 antimalarial compounds available through the NCI compound library or Sigma Chemical Company (quinidine quinine, primaquine, chloroquine, hydroxychloroquine, halofantrine, mefloquine), 2 inactive quinolines as negative controls (quinoline, quinolinic acid), and trichostatin acid, a known HDAC1 inhibitor, as the positive control. The antimalarials caused differentiation of breast tumor cell lines and increased histone H4 acetylation. Only halofantrine, a non-quinoline antimalarial may directly inhibit HDAC1 enzyme activity. We published that quinidine, a quinoline antimalarial, acts via a novel mechanism causing differentiation of breast tumor cells following a transient proteolytic degradation of th HDAC1 protein. Our screen has been extended to include 17 additional NCI compounds that possess a quinoline ring; their anticancer mechanisms are unknown, but we have preliminar evidence that several cause breast tumor differentiation. Analysis of these 27 compounds by molecular modeling is not yet complete.

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Text Annual Report 01

INTRODUCTION:

Histone deacetylase (HDAC) enzymatically removes acetyl moieties form the epsilon-amino of L-lysine in histone proteins. Histone modifications such as deacetylation participate in the modulation of gene expression. Drugs that inhibit HDAC activity have anti-tumor activity in transformed cell lines, in animal tumor models and in clinical trial. HDAC inhibitory drugs constitute a potentially important new category of antitumor agents. It is our goal to determine if HDAC inhibitors cause growth inhibition and cell death in human breast tumor cell lines and to identify new HDAC inhibitors for therapeutic use. Our approach is based upon a fortuitous observation that quinidine and quinine, two quinoline antimalarials, caused growth inhibition, differentiation and cell death in MCF-7 human mammary carcinoma cells. We have now undertaken a screen of antimalarial drugs to establish structure activity relationship for HDAC1 inhibition. Additional compounds that possess both antitumor activity in the NCI drug discovery program and a quinoline ring structure have been included in this screen. The significance of this work is that it provides the basis for developing new drug therapies for breast cancer that cause tumor cell differentiation with minimal cytotoxicity to normal tissues.

BODY:

Task 1 A total of 27 compounds have been obtained for the CoMFA analysis. Eleven of the 15 compounds described in the proposal were quinolines. Quinacrine proved too toxic and has been dropped. Amodiaquine and hydroxychloroquine were added. Quininone and quinocide were not obtainable from NCI, nor were we successful in multiple attempts to obtain additional antimalarial compounds from Walter Reed Army Medical Center. Instead, additional quinoline compounds were obtained from the NCI, Division of Experimental Therapeutics. A minimum of 25 compounds is required for CoMFA.

The PI completed Pharmacy 491C, Introduction to Molecular Modeling (3CR). This was a computer lab intensive course designed to teach computational chemistry pitfalls and applications. Molecular mechanics and molecular dynamics were covered in Amber; Insight II was used for analyzing protein structure and protein-small molecule docking. The PI has continued to develop skills necessary to perform conformational molecular field analysis, a topic requiring the use of Sybyl. I have used this program, but have not completed the energy minimizations on the 27 compounds we have identified for our screen. I have experienced difficulty converting the chemical structures of these compounds available through the NCI website into a format that is read by the Sybyl program. I have drawn a few compounds by hand in Sybyl, and performed energy minimization; this process is inefficient but workable. I have an excellent starting point for the energy minimizations using the solvated X-ray crystallographic structure of quinidine and quinine that was provided by the Cambridge Crystallographic Data Centre, Cambridge, UK.

The central goal is the development of a molecular model of a new class of HDAC1 inhibitors. The end-point chosen for this analysis is critical to the value of the predictive model, and the following was established as a strategy for end-point analysis. The quinoline compounds were assigned a rank order on the basis of the IC50 for mitochondrial metabolism of a tetrazolium dye (MTS assay). This assay is usually referred to as a cell viability assay because it is an indirect measure of the ability of a cell to generate ATP needed for cell function. We screened all the compounds in two breast tumor cell lines: the more slowly growing, ER-positive MCF-7 cells and the rapidly growing, ER-negative MDA-MB-231 cell line. Using the MTS IC50 values compounds were screened for differentiating activity in both cell lines using the Oil Red O lipid droplet stain. Complete data was obtained on 16 of the 27 compounds at the time of this report. These data are described in Table 1. Work on the remaining 11 compounds is in progress.

Task 2 Compounds that tested positive for differentiation (Oil Red O positive) at or near the MTS IC50 were screened for direct inhibition of HDAC enzymatic activity in a cell free assay. The original proposal stated that Dr. Jim Davie would conduct the initial HDAC assays, because of his expertise with the radioactive assay. Recently, a fluorescence based cell-free assay kit became commercially available through Bio-Mol. This assay was used to screen the quinoline compounds for inhibition of HDAC1 from

human HeLa cell extracts by an undergraduate summer research student, Mr. Andrew Freeman. Of the 16 compounds tested, only one compound NSC#3852 was a direct HDAC1 inhibitor. Trichostatin Acid (TSA) was used as the positive control (IC 50= 8.5 nM). This result was not that surprising because earlier work suggested that differentiation in the breast tumor cells by quinidine was the result of a novel mechanism that resulted in histone hyperacetylation without direct inhibition of HDAC enzyme activity (see J.Biol.Chem. publication in the appendix for detail.)

Task 3 The PI recruited a postdoctoral fellow, Dr. Anna Martirosyan. Dr. Martirosyan began work in this laboratory in August, 2000. Dr.Martirosyan has conducted the MTS and Oil Red O screening of many of the NCI compounds. Two graduate students in the laboratory, Dr. Qun Zhou and Ms. Rayhana Rahim, have also participated in the compound screening.

Task 4 A nucleosome release ELISA was used as a quantitative index of apoptotic activity. At the MTS IC50, only one of nine quinolines (test results to date) caused apoptosis. Etoposide was used as the positive control. In this small subset of compounds that included the antimalarials (quinidine, quinine, amodiaquine, primaquine, chloroquine, halofantrine, mefloquine, quinoline and quinolinic acid), induction of apoptosis required higher drug concentrations than those that affected MTS metabolism or cellular differentiation. We also observed that low concentrations of trichostatin acid (TSA), a classic HDAC inhibitor caused direct inhibition of HDAC activity (IC50= 8.5 nM) and inhibition of MTS metabolism (IC50 in MCF-7 cells = 35nM), but higher concentrations (100-400nM) were required to induce apoptosis by 48 hours in MCF-7 cells. We conclude that apoptosis induction measured using this assay will not be a suitable end-point for our CoMFA analysis.

Task 4 also includes assessment of histone hyperacetylation by drug treatment. We have established qualitatively that quinidine, quinine, amodiaquine, primaquine and chloroquine raise levels of acetylated histone H4 in MCF-7 cells. To use histone H4 acetylation as an end-point in the CoMFA, requires that a quantitative measure of histone acetylation be found. We have worked towards developing methods to quantitatively measure the increase in acetylated H4. There are a number of problems in finding a quantitative way to measure histone acetylation. 1) We found the quality of the histone H4 antibody available varies among commercial vendors and from a single vendor, lot –to –lot. 2) We found that histones do not transfer efficiently from the gel onto nylon filters. 3) We thought we could normalize the signal from the acetylated H4 antibody to total histone on the nylon filter, using antibodies that recognize total histone H4 on the blot (both the acetylated and the non-acetylated form). There appear to be differences in the efficiency of transfer of the unacetylated histone vs. acetylated histone from the gel onto the Nylon membrane or the quality of the antibodies. The signal from the acetylated histones exceeded that of the total histone signal. We are trying other approaches to quantitatively measuring levels of histone acetylation in reponse to drugs using nitrocellulose membranes, and using the stained membrane as a measure of total histone on the blotting membrane. We are also pursuing a collaborative arrangement to develop a mass spectrometry assay to quantitatively measure histone acetylation.

New Experiments Proposed for Task 4: To complete Task 4 we propose to investigate two additional end-points that are representative of drugs that cause differentiation: 1) loss of Ki67 antigen expression which is a marker for eells that exit the cell cycle and enter G0 (48 hours) and 2) induction of the G1 cyclin-dependent kinase inhibitor, p21/WAF1/Cip1 using a commercial ELISA kit. Methods for both end-points are straightforward and readily lend themselves to quantitative analysis. These assays may prove to be suitable for the CoMFA if quantitative analysis of histone H4 acetylation eludes us.

Task 5 We request that task 5 and research goal 2 as originally proposed be eliminated from further consideration. It was brought to my attention that some investigators have had difficulty demonstrating HDAC activity from the baculovirus expressed HDAC enzyme. In addition, most of our data suggest that the quinoline drugs are not direct HDAC inhibitors. Thus, it is possible that the drugs do not directly bind HDAC.

New Experiments Proposed for Task 5 (Goal 2): The mechanism of quinidine induction of histone H4 hyperacetylation is sensitive to proteasome inhibitors (JBC publication in the appendix). We therefore propose to examine if HDAC undergoes ubiquitination and whether quinidine regulates this process.

Task 6 After screening with MCF-7 and MDA-MB-231 cells, we will screen for selective tumor cell toxicity using normal HMEC and immortalized MCF10A cells.

Task 7 We request that task 7 be dropped from further consideration: quinacrine mustard is too toxic, and we do not think the quinolines are direct HDAC inhibitors.

Task 8 We are preparing one manuscript describing histone hyperacetylation, cell differentiation, apoptosis in MCF-7 cells by the quinoline antimalarials. We are preparing an abstract for submission August 1 to the American Society for Cell Biology.

KEY RESEARCH ACCOMPLISHMENTS:

*Established that quinoline antimalarial agents have differentiating and antiproliferative activity in human breast tumor cell lines.

*Chloroquine and Hydroxychloroquine (Plaquenil) cause differentiation and inhibition of growth in MCF-7 and MDA-MB-231 human breast tumor cells at concentrations that are non-toxic to immortalized, non-transformed MCF10A human breast cells.

*Established that quinoline antitumor compounds NSC#4238, 4239, 10010, 2039 and 3852 show promise as differentiating agents in human breast tumor cell lines.

- Identified NSC#3852 as a direct inhibitor of HDAC1 enzyme activity in vitro.
- Using the MTS cell viability assay as an indication of antitumor activity in MCF-7 and MDA_MB_231 cells, all of the quinoline antitumor agents examined were more potent than what is reported in the NCI web site (LC50 and IG50).

REPORTABLE OUTCOMES:

*One published manuscript, Zhou Q, Melkoumian ZK, Lucktong A, Moniwa M., Davies JR and Strobl, JS. J. Biol. Chem. 275: 35256-35263 (2000).

*Research support for 1 postdoctoral fellow and two graduate students.

*Funded grant application, WVU Research Incentive Competitive Grants Program, $17,500 to establish an Apoptosis Analysis Facility.

CONCLUSIONS:

We conclude that the antimalarial quinoline compounds warrant re-investigation of their pharmacologic activity as antitumor agents in breast cancer. Although these compounds show low potency in comparison with some other tumor differentiating agents, our preliminary studies indicate that the antimalarials show selective toxicity to breast tumor cells compared with normal breast epithelial cells. The "antimalarials" have potential both as antitumor and as chemopreventative agents.

We conclude that some of the quinoline antitumor agents listed in the NCI library may act as tumor differentiating agents. A very modest search revealed one compound that was a direct HDAC inhibitor and thus a potential tumor differentiating agent.

Differentiation therapy holds promise as a less toxic alternative to traditional chemotherapeutic regimes and as a mode of chemoprevention. The identification of quinolines as low potency, low toxicity differentiating agents may enable us to develop new agents to control tumors by promoting tumor cell differentiation.

Table 1. BREAST TUMOR DIFFERENTIATING QUINOLINES

		Growth End-Points		Differentiation End-Points			Proposed	Proposed
RANK	**NSC #**	**WVU**	**NCI**	**ORO**	**Histone**	**$HDAC_{activity}$**	**p21/WAF**	**Ki67**
		IC50	**GI50**	**Lipid Stain**	**H4-Acety.**	**Inhibitor**		
		MTS	**SRB**					
		(uM)	**(uM)**					
		Cell Line	Cell Line	Cell Line	Cell Line	HeLa X-tract		
		1 2	1 2	1 2	1 2			
1	86372	.14 .17	.12 .16	no		no		
2	4238	.31 .88	1.6 .85	yes		no		
3	4239	.60 .39	2.3 5.9	yes		no		
4	146397	.88 1.8	1.1 2.6	no		no		
5	**149765**	3.4 >100	16.4 43.7	yes no	yes	no		
6	**157387**	3.0 nd	nd nd	yes yes		no		
7	85701	2.8 .46	4.2 nd	no		no		
8	10010	6.5 .72	5.4 1.27	yes		no		
9	85700	6.5 nd	7.4 33.2					
10	**Amodiaquin**	6.9 nd	nd nd	yes yes	yes	no		
11	86371	7.6 nd	nd nd					
12	2039	12.7 7.3	1.7 3	weak		no		
13	86373	13.5 nd	nd nd					
14	69603	14.1 nd	.85 .57					
15	305819	19 nd	12.6 9.4					
16	QuinolinicAcid	28.2 nd	nd nd	weak		no		
17	**5362**	40.1 nd	60 100	yes yes	yes	no		
18	**14050**	48.2 51.2	19 16	yes yes	yes	no		
19	**305789**	58.9 nd	10.2 2.5	yes yes		no		
20	3852	61.2 nd	1.8 6.4	weak		yes		
21	Quinoline	61.5 nd	nd nd			no		
22	**Hdrxy/clq**	71.2 43.6	nd nd	yes yes		no		
23	249913	102 nd	nd nd					
24	**Quinidine**	113.6 nd	nd nd	yes yes	yes	no		
25	15783	308? Nd	4.3 16.1					
26	339004	Inactive	45.3 70					
27	124637	Not Tested	13.6 17					

Legend Compounds are ranked based on growth inhibition in the MTS (tetrazolium dye assay) and the SRB(sulforhodamine dye assay). Compounds in **bold** are antimalarial drugs.
149765 = Primaquine; 157387 = Mefloquine; 5362 = Quinine; 14050 = Chloroquine;
305789 = Halofantrine
Cell line 1 = MCF-7 Cell line 2 = MDA-MB-231. nd= not determined
ORO = Oil Red O Histone H4 Acetylation was measured by Western blot
HDAC activity was measured using BioMol Fluor d Lys assay kit.

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Rapid Induction of Histone Hyperacetylation and Cellular Differentiation in Human Breast Tumor Cell Lines following Degradation of Histone Deacetylase-1*

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Quinidine inhibits proliferation and promotes cellular differentiation in human breast tumor epithelial cells. Previously we showed quinidine arrested MCF-7 cells in G_1 phase of the cell cycle and led to a G_1 to G_0 transition followed by apoptotic cell death. The present experiments demonstrated that MCF-7, MCF-7*ras*, T47D, MDA-MB-231, and MDA-MB-435 cells transiently differentiate before undergoing apoptosis in response to quinidine. The cells accumulated lipid droplets, and the cytokeratin 18 cytoskeleton was reorganized. Hyperacetylated histone H4 appeared within 2 h of the addition of quinidine to the medium, and levels were maximal by 24 h. Quinidine-treated MCF-7 cells showed elevated $p21^{WAF1}$, hypophosphorylation and suppression of retinoblastoma protein, and down-regulation of cyclin D1, similar to the cell cycle response observed with cells induced to differentiate by histone deacetylase inhibitors, trichostatin A, and trapoxin. Quinidine did not show evidence for direct inhibition of histone deacetylase enzymatic activity *in vitro*. HDAC1 was undetectable in MCF-7 cells 30 min after addition of quinidine to the growth medium. The proteasome inhibitors MG-132 and lactacystin completely protected HDAC1 from the action of quinidine. We conclude that quinidine is a breast tumor cell differentiating agent that causes the loss of HDAC1 via a proteasomal sensitive mechanism.

Histone deacetylase (HDAC)[1] proteins comprise a family of related proteins that act in conjunction with histone acetyltransferase proteins to modulate chromatin structure and transcriptional activity via changes in the acetylation status of histones. Histones H3 and H4 are the principal histone targets of HDAC enzymatic activity, and these histones undergo acetylation at lysine residues at multiple sites within the histone tails extending from the histone octamer of the nucleosome core. The association of HDAC proteins with mSin3, N-CoR, or SMRT and other transcriptional repressors has led to the hypothesis that HDAC proteins function as transcriptional corepressors (reviewed in Ref. 1). The spectrum of genes that show alterations in gene transcription rates in response to decreased HDAC activity is quite restricted (2). Yet, small molecule inhibitors of the enzyme histone deacetylase (HDAC) such as trichostatin A (TSA), superoylanilide hydroxamic acid (SAHA), trapoxin, and phenyl butyrate cause major alterations in cellular activity including the induction of cellular differentiation and apoptosis (3–5). Trichostatin A, SAHA, and trapoxin stimulate histone acetylation by acting as direct inhibitors of HDAC enzyme activity (6). Trichostatin A, SAHA, and trapoxin possess lysine-like side chains and act as chemical analogs of lysine substrates. Molecular models based upon the x-ray crystal structure of an HDAC-like protein indicate that trichostatin A and SAHA can bind within the active site of the HDAC enzyme and interact with a zinc metal ion within the catalytic pocket that is critical for enzymatic activity (7). Trapoxin is an irreversible HDAC enzyme inhibitor (8).

Much remains to be learned about the biochemical events subsequent to HDAC inhibition that lead to cell cycle arrest, cellular differentiation, and apoptosis. However, a spectrum of biological responses characteristic of HDAC inhibitors has emerged, including cell cycle arrest in G_1, elevated $p21^{WAF1}$ expression, hypophosphorylation of retinoblastoma protein (pRb), hyperacetylation of histones, particularly H3 and H4, and apoptosis. Histone hyperacetylation is directly linked to the activation of p21 transcription and is p53-independent (5). This observation provides an important link between HDAC inhibition and cell cycle arrest because $p21^{WAF1}$ plays a critical role in causing G_1 cell cycle arrest via inhibition of the G_1 cyclin-dependent kinase family (9). Overexpression of $p21^{WAF1}$ has also been associated with apoptosis, but the mechanism of $p21^{WAF1}$ induction of apoptosis requires further investigation (10).

Cancer therapy that targets the activity of genes or gene products controlling cell cycle progression, differentiation, and apoptosis is a promising new strategy. Because HDAC inhibitors regulate the cell cycle and cause both cellular differentiation and apoptosis, they comprise an interesting group of compounds with potential for development into a new category of clinically significant anti-tumor agents. Single, key protein targets for "gene-regulatory chemotherapy" are difficult to identify due to the existence of parallel, functionally overlap-

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[1] The abbreviations used are: HDAC, histone deacetylase; DMEM, Dulbecco's modified Eagle's medium; FBS, fetal bovine serum; MG-132, carbobenzoxy-L-leucyl-L-leucyl-L-leucinal; PBS, phosphate-buffered saline; pRb, retinoblastoma protein; SAHA, superoylanilide hydroxamic acid; TSA, trichostatin A; HRP, horseradish peroxidase; HMEC, human mammary epithelial cells; ER, estrogen receptor; MTS, 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2*H*-tetrazolium; CDK, cyclin-dependent kinase.

ping signaling cascades. For this reason, use of cancer therapies that target multiple intracellular signaling pathways, such as observed with the HDAC inhibitors, is an intriguing approach that addresses the problem of redundancy in growth signaling pathways. In this regard, the HDAC inhibitor phenyl butyrate was recently shown to have clinical anti-tumor activity (11).

Quinidine is a natural product therapeutic agent originally used as an anti-malarial and as an anti-arrhythmic agent. Previous studies with human breast tumor cell lines demonstrated that quinidine (90 μM) is an anti-proliferative agent as well. Quinidine arrested cells in early G_1 phase and induced apoptosis by 72–96 h in MCF-7 cells (12), but the biochemical basis for the anti-proliferative effect of quinidine was not well understood. To clarify the molecular mechanisms of the anti-proliferative activity of quinidine, we investigated the effects of quinidine on histone acetylation and cell cycle regulatory proteins. In this report, we show that quinidine causes hyperacetylation of histone H4, down-regulation of HDAC1 protein levels, and cellular differentiation in a panel of human breast tumor cell lines. We conclude that quinidine is a novel differentiating agent that stimulates histone hyperacetylation as a result of HDAC1 protein degradation.

MATERIALS AND METHODS

Cell Culture—Permanent cell lines derived from patients with breast carcinomas were used in these studies. MCF-7 cells, passage numbers 40–55, MCF-7*ras* (13), T47D, MDA-MB-231, and MDA-MD-435 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) (BioWhittaker, Walkersville, MD) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (HyClone Laboratories, Inc., Logan, Utah), 2 mM glutamine, and 40 μg/ml gentamicin. Experiments were performed in this medium supplemented with 5% FBS. The cells were maintained at 37 °C in a humidified atmosphere of 93% air, 7% CO_2. After 6 days, cells became about 70–80% confluent and were passaged at a 1:5 ratio (MCF-7) or at a 1:10 ratio (all others). Normal human mammary epithelial cells (HMEC) were obtained from Clonetics, San Diego, CA, and were grown according to directions of the suppliers. Cells were grown from frozen stocks and used for 1–3 passages. Quinidine, TSA, and all-*trans*-retinoic acid were purchased from Sigma. The cell-permeant proteasome inhibitors, MG-132 and lactacystin, were purchased from Calbiochem.

Growth Inhibition Assays—Growth inhibition by cell numbers was assayed by plating cells in 35-mm^2 dishes ($1–1.5 \times 10^5$) containing DMEM, 5% FBS plus quinidine (90 μM). Viable cells were counted using a hemocytometer, and trypan blue (0.02%) exclusion was used as an indicator of viability. Cell growth was also monitored in a 96-well plate format using the One Solution Cell Proliferation Assay (Promega, Madison, WI), which is based upon metabolic bioreduction of a tetrazolium compound (Owen's reagent) to a colored formazan product that absorbs light at 490 nm. The plating density for the 96-well dishes (cells/well) was varied depending upon the relative growth rates of the cell lines as follows: HMEC (2000), MCF-7 (1000), MDA-MB-231 (500), T47D (1500), and MCF-7*ras* (500). The One Cell Proliferation Assay Reagent was added to each well and incubated for 2 h at 37 °C. Absorbance (490 nm) was read using a Molecular Devices PC340 (Sunnyvale, CA).

Microscopic Imaging—Cells were plated (1×10^5) on sterile coverslips in 35-mm^2 dishes and grown for 96 h in DMEM, 5% FBS supplemented with either 10 μM all-*trans*-retinoic acid (in 0.01% ethanol) or 90 μM quinidine (in H_20). Control cells were grown in medium containing a final concentration of 0.01% ethanol. The presence of ethanol had no effect upon lipid droplet accumulation compared with cells grown in DMEM, 5% FBS. Cells were fixed in 3.7% formaldehyde/PBS, rinsed in PBS (PBS: 140 mM NaCl, 2 mM KCl, 80 mM $Na_2HPO_4 \cdot 7H_2O$, 1.5 mM KH_2PO_4, pH 7.0), then treated briefly with 0.4% Triton X-100 in PBS. After rinsing three times in PBS, the cells on coverslips were incubated for 30 min at 37 °C with a primary antibody to cytokeratin 18 (1:1 dilution, provided by Dr. Guillaume van Eys, Maastricht University), rinsed, and incubated (30 min/37 °C) with Texas Red conjugated secondary antibody (goat anti-mouse IgG, Sigma). Alternatively, cells were incubated with fluorescein-phalloidin (1:200 dilution of a-5 μg/0.1 ml solution, Sigma) in the dark for 40 min at room temperature, rinsed, and incubated for 5 min (room temperature) with the fluorescent lipid stain, Nile Red (1:10,000 dilution of a 1 mg/ml acetone solution, Sigma) (14–15). All coverslips were rinsed in PBS and mounted with Fluoromount-G containing 2.5% *N*-propyl galate. Images were obtained using a Zeiss Axiovert 100 M confocal microscope (× 63 objective).

Immunoblotting—Cells were harvested from confluent T-75 flasks and subcultured (1×10^6) in 60-mm^2 dishes. On subcultivation, this confluent population of cells (85% in G_1) synchronously proceeded through the cell cycle. To prepare whole cell lysates, the cells were harvested at the times indicated by scraping into ice-cold buffer (50 mM Tris-HCl, 0.25 M NaCl, 0.1% (v/v) Triton X-100, 1 mM EDTA, 50 mM NaF, and 0.1 mM Na_3VO_4, pH 7.4). Protease inhibitors (protease inhibitor mixture, Roche Molecular Biochemicals) were added immediately. Cell lysates were centrifuged in an Eppendorf microcentrifuge (14,000 rpm, 5 min) at 4 °C, and the supernatants were used in immunoblotting experiments.

Histones were prepared from cells grown at a density of 1×10^7/T-162 flask. To harvest the cells, the flasks were placed on ice, and the growth medium was removed. Following a quick rinse with ice-cold PBS, cells were scraped into 1 ml of ice-cold lysis buffer (10 mM Tris-HCl, 50 mM sodium bisulfite, 1% Triton X-100 (v/v), 10 mM $MgCl_2$, 8.6% sucrose, pH 6.5) and nuclei released by Dounce homogenization. The nuclei were collected by centrifugation (3,000 rpm, 10 min, SS-34 rotor) and washed three times with the lysis buffer. Histones were extracted from the crude nuclear pellets using the procedure of Nakajima *et al.* (16). The pellets were resuspended in 0.1 ml of ice-cold sterile water using a vortex and concentrated H_2SO_4 to 0.4 N was added. The preparation was incubated at 4 °C for 1 h and then centrifuged (17,000 rpm, 10 min, Sorvall SS-34 rotor). The supernatant containing the extracted histones was mixed with 10 ml of acetone, and the precipitate was obtained after an overnight incubation at −20 °C, collected, and air-dried. The acid-soluble histone fraction was dissolved in 50 μl of H_2O and stored at −70 °C.

The protein concentration of the whole cell lysate supernatants or histone preparations was determined using the BCA protein assay (Pierce) and bovine serum albumin as a standard. Equal amounts of protein were loaded onto SDS-polyacrylamide gels. Molecular weights of the immunoreactive proteins were estimated based upon the relative migration with colored molecular weight protein markers (Amersham Pharmacia Biotech). Proteins were transferred to polyvinylidene difluoride membranes (NOVEX, San Diego, CA) and blocked at 4 °C using 5% nonfat milk blocking buffer (1 M glycine, 1% albumin (chicken egg), 5% non-fat dry milk, and 5% FBS) overnight. The membranes were incubated with primary antibodies for 3 h at room temperature. The antibody sources were as follows: mouse monoclonal anti-p27 (F-8, SC-1641), rabbit polyclonal anti-CDK4 (C-22), goat polyclonal anti-HDAC1 (N-19, SC-6299), all from Santa Cruz Biotechnology (Santa Cruz, CA); mouse monoclonal anti-pRb (14001A) from PharMingen (San Diego, CA); mouse monoclonal anti-cyclin D1 (NCL-cyclin D1, 113105) from Novocastra (Burlingame, CA); mouse monoclonal anti-p16 (Ap-1), p21 (WAF1,Ap-1), p53 (Ap-6) from Calbiochem; and anti-acetylated histone H4 antibody (rabbit polyclonal, Upstate Biotechnology Inc.). The primary antibodies were diluted at 1:500 in Western washing solution (0.1% non-fat dry milk, 0.1% albumin (chicken egg), 1% FBS, 0.2% (v/v) Tween 20, in PBS, pH 7.3). The antigen-antibody complexes were incubated for 1 h at room temperature with horseradish peroxidase (HRP)-conjugated secondary antibodies (goat IgG-HRP (SC-2020), rabbit IgG-HRP (SC-2004), or mouse IgG-HRP (SC-2005) from Santa Cruz Biotechnology) at a final dilution of 1:3000 in Western washing solution. After washing three times with Tris-buffered saline (10 mM Tris-HCl, pH 7.5, 0.5 M NaCl, and 0.05% (v/v) Tween 20), antibody binding was visualized using enhanced chemiluminescence (SuperSignal West Pico, Pierce) and autoradiography.

In Vitro HDAC Activity Assay—Quinidine HCl was added to a chicken erythrocyte cellular extract, which contained HDAC activity, at concentrations of 90 μM (18). HDAC assays were performed as described in Hendzel *et al.* (17). Briefly, the cellular extract was incubated with 500 μg of acid-soluble histones isolated from [^{3}H]acetate-labeled chicken erythrocytes for 60 min at 37 °C. Reactions were terminated by addition of acetic acid/HCl to a final concentration of 0.12/0.72 N. Released [^{3}H]acetate was extracted using ethyl acetate and quantified by scintillation counting. Samples were assayed three times, and the non-enzymatic release of label was subtracted to obtain the reported values.

RESULTS

Hyperacetylation of Histone H4—Antibodies that recognize acetylated forms of histone H4 have been used as a probe for agents that cause histone hyperacetylation (19). In Western blot experiments, we compared the ability of quinidine to cause

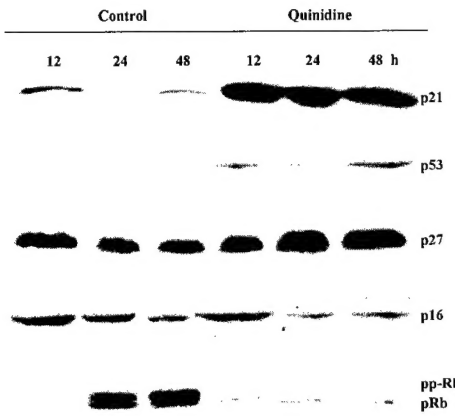

FIG. 3. **G_1 cell cycle proteins in MCF-7 cells.** Cells released from confluency were plated into control medium or medium containing 90 μM quinidine. Whole cell lysates were prepared 12, 24, or 48 h after plating and assayed by immunoblotting for the cyclin-dependent kinase inhibitors, $p21^{WAF1}$ ($n = 3$), p27 ($n = 1$), p16 ($n = 3$), and p53 ($n = 3$) after electrophoresis of 50 μg of protein/lane through 12% SDS-polyacrylamide gels. pRb protein was immunoprecipitated from 500 μg of whole cell lysate protein using an antibody that recognizes phosphorylated and non-phosphorylated pRb (56). This entire immunoprecipitate was electrophoresed in a 7.5% SDS-polyacrylamide gel and immunoblotted using this same antibody. Results shown are typical of two independent analyses.

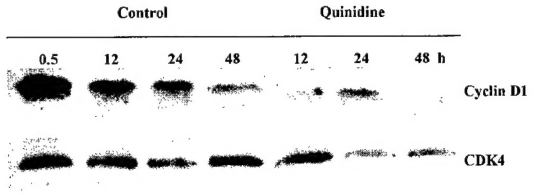

FIG. 4. **Cyclin D-CDK4 in MCF-7 cells.** Confluent MCF-7 cells were subcultured in control medium or medium containing 90 μM quinidine. Whole cell lysates were prepared 0.5, 12, 24, and 48 h after subculture. Equal protein aliquots (50 μg/lane) were electrophoresed in 12% SDS-polyacrylamide gels and assayed for cyclin D1 and CDK protein levels by immunoblotting. Results shown are representative of three independent experiments.

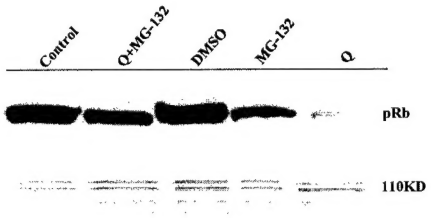

FIG. 5. **Proteasome inhibitor modulates retinoblastoma protein levels.** Confluent MCF-7 cells were subcultured in the presence of 90 μM quinidine, 30 μM MG-132, or quinidine + MG-132 for 24 h, then harvested, and whole cell extracts (100 μg/lane) were analyzed for pRb. A Coomassie Blue-stained protein is shown as the loading control.

A small, less than 2-fold increase in p27 levels was observed in cells exposed to quinidine for 24–48 h, whereas levels of p16 were unchanged (Fig. 3). Quinidine treatment decreased cyclin D1 and CDK4 protein levels after 12 h of treatment (Fig. 4), indicating that the cyclin-dependent kinase inhibitor, $p21^{WAF}$, as well as an important G_1 phase target of $p21^{WAF1}$, the cyclin D1-CDK4 complex, are early targets of quinidine in MCF-7 cells. This profile of activity is consistent with the observed cell cycle arrest of quinidine-treated MCF-7 cells in mid-G_1 phase (12).

In MCF-7 cell extracts probed using anti-pRb antibodies, two separate but closely migrating bands were distinguishable. The upper band contained more highly phosphorylated pRb, and the lower band contained unphosphorylated or hypophosphorylated pRb. Control cells showed a faint pRb signal at 12 h, typical of cells in early G_1 phase, and increased expression of both phosphorylated and unphosphorylated pRb at 24 and 48 h. Quinidine-treated MCF-7 cells had no detectable hyperphosphorylated pRb at any time point examined, and total levels of pRb protein failed to increase with progression through G_1 phase as seen in the control, proliferating cells (Fig. 3). The decrease in pRb phosphorylation level was predictable based on the increase in $p21^{WAF1}$ and decreased levels of both cyclin D1 and CDK4 (Fig. 4). In addition, Nakanishi *et al.* (24) showed that $p21^{WAF1}$ can bind pRb protein and block its phosphorylation. However, the actions of quinidine upon $p21^{WAF1}$ and cyclin D-CDK4 activity do not explain why the levels of total pRb protein were so low. Reductions in the cellular content of phosphorylated pRb protein in MCF-7 cells by quinidine is an important additional level of cell cycle control that effectively attenuates progression of cells out of G_1 phase and has been reported in other tumor cell lines in response to HDAC inhibition (22). In Fig. 5 we show data suggesting that the 26 S proteasome pathway regulates the total pRb content. MCF-7 cells incubated for 24 h in MG-132 or MG-132 plus quinidine had more total pRb than cells incubated with quinidine alone. Thus, quinidine promoted the loss of both HDAC1 and pRb, and inhibition of the 26 S proteasome pathway restored the levels of both of these proteins to that seen in the untreated cells. We have no direct evidence that quinidine promotes the proteasomal degradation of either protein. We hypothesize that quinidine may direct degradation of HDAC1 by the proteasome or, alternatively, quinidine might stimulate the proteasomal degradation of other regulatory factor(s) that act to maintain HDAC1 and pRb protein levels.

MCF-7 cells express wild-type p53 protein. Normal p53 is a short lived protein that is maintained at low levels, but in response to cell stress or DNA damage, p53 is stabilized and accumulates in the nucleus where it functions as a transcription factor inducing $p21^{WAF}$, G_1 cell cycle arrest, and apoptosis (25). Wild-type p53 down-regulates pRb levels in MCF-7 cells (26). Although Saito *et al.* (22) showed that p53 is not required for pRb down-regulation by HDAC inhibitors in all cell lines, quinidine-treated MCF-7 cells have elevated p53 levels (5–7-fold) (Fig. 3). Thus, p53 could contribute to the maintenance of the G_1 cell cycle arrest in MCF-7 by sustaining $p21^{WAF1}$ protein levels and suppressing pRb protein levels.

Growth Arrest and Cellular Differentiation in Human Breast Tumor Cell Lines—In contrast to MCF-7 cells, human breast tumor cell lines T47D, MDA-MB-231, and MDA-MB-435 express p53 proteins with distinct point mutations (27). To test for a requirement of p53, this panel of human breast tumor cell lines was exposed to quinidine, and the effects of quinidine on cell growth were compared (Fig. 6). The data shown are viable cell numbers/well, bioreductive metabolism/well, or both. In all four cell lines growth was suppressed in a concentration-dependent fashion between 10 and 90 μM quinidine, and maximal growth inhibition was observed at ~90 μM quinidine (data not shown). These data showed that growth suppression by quinidine is a p53-independent response. It is interesting that quinidine was not overtly cytotoxic in HMEC, a line of normal human mammary cells (28).

Evidence that quinidine elicited cellular differentiation in MCF-7 human breast tumor cells in conjunction with the inhibition of cell growth was obtained using maximally effective concentrations of quinidine or retinoic acid (data not shown). Antibodies directed against cytokeratin 18 (29) were used to probe the organization of the cytoskeleton (Fig. 7). In these studies, all-*trans*-retinoic acid (10 μM) was used to compare the differentiation response (30). Control MCF-7 cells showed expression of cytoplasmic cytokeratin 18 in a disorganized fash-

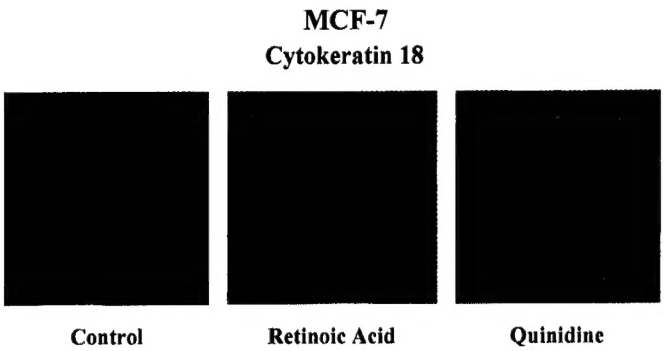

FIG. 7. **Cytokeratin 18 in MCF-7 cells.** Cells were replica-plated (2×10^5) on sterile coverslips in 35-mm^2 dishes in medium containing 0.01% ethanol (control), 10 μM retinoic acid, or 90 μM quinidine and grown for 96 h. Cytokeratin 18 detection using a Texas Red-tagged secondary antibody is shown using confocal microscopy. Data shown are typical fields representative of two independent experiments.

lines and increased by retinoic acid and quinidine. Lipid droplet accumulation was more marked in all four cell lines treated with quinidine than with retinoic acid. These data demonstrate that induction of a more differentiated phenotype is a general response of human mammary tumor cells to quinidine.

Hyperacetylation of Histone H4 in Mammary Tumor Cell Lines by Quinidine—To determine whether differentiation and histone acetylation were linked, we investigated the histone H4 acetylation status of quinidine-treated T47D, MDA-MB-231, and MCF-7$_{ras}$ cells. MCF-7, MCF-7*ras*, T47D, and MDA-MB-231 cells were incubated for 24 h in the presence or absence of quinidine, and then histones were extracted for immunoblotting. Fig. 9 shows that histone H4 was hyperacetylated in all cell lines treated with quinidine. Control cells contained no hyperacetylated histone H4.

DISCUSSION

Quinidine-induced histone H4 hyperacetylation in MCF-7 human breast carcinoma cells can be attributed to the rapid elimination of HDAC1 protein, a response that was blocked by MG-132 and lactacystin, two inhibitors of proteasome-mediated proteolysis. HDAC1 protein was undetectable within 30 min after the addition of quinidine to the medium of MCF-7 cells, and hyperacetylated histone H4 appeared between 1 and 2 h. Levels of HDAC1 protein were completely suppressed between 0.5 and 6 h, and during this time H4 acetylation levels increased. H4 acetylation was maintained at 12 and 24 h, despite the partial restoration of HDAC1 protein at these same time points. These data indicate that quinidine-induced reductions in HDAC1 protein levels are unlikely to explain fully the regulation of H4 acetylation state in MCF-7 cells by quinidine. Additional HDAC enzymes or effects upon histone acetylation rates could possibly play a role as well.

An earlier study showed that over this initial 48-h period, 80% of the MCF-7 cell population had shifted into G_0, a quiescent state marked by the absence of Ki67 antigen immunoreactivity (12). Cellular differentiation manifested as the accumulation of lipid droplets, and a reorganization of the cytokeratin 18 cytoskeleton was evident after this initial 48-h period. Quinidine exhibited all the responses typical of known HDAC inhibitory drugs, with the exception that quinidine had no direct inhibitory effect upon HDAC1 enzymatic activity. We conclude from the current studies that quinidine is a novel differentiating agent that causes histone hyperacetylation, in part, by physical elimination of HDAC1 protein rather than the inhibition of HDAC enzymatic activity.

Histone H4 hyperacetylation and induction of cellular differentiation by quinidine were seen in a panel of human breast tumor cell lines that were selected for study on the basis of their diversity of genetic backgrounds. The differentiation response to quinidine was independent of the estrogen receptor (ER) status. Cell lines representative of ER-positive and ER-negative human breast carcinoma cells were induced to differentiate in the presence of quinidine. The ER status of the estrogen receptor positive cell lines is MCF-7 (ER-α and ER-β), T47D (ER-α and ER-β), and MDA-MB-231 (ER-β). MDA-MB-435 cells expressed very low levels of ER-β and no ER-α (37, 38). MCF-7 and T47D cells display an epithelial morphology and show similarities with mammary ductal and luminal epithelial cells, respectively (30, 39). MDA-MB-231 cells exhibit an elongated cellular morphology that is also typical of MDA-MB-435 cells. Our results demonstrate that quinidine is a differentiation agent in both types of mammary tumor cells.

HDAC inhibitors reverse the transformed phenotype of NIH3T3*ras* cells, and this property has been used successfully as a screening assay for the identification of new HDAC inhibitors (40, 41). Quinidine elicited a more differentiated phenotype in MCF-7*ras* cells, an MCF-7 cell derivative produced by stable transformation with v-Ha-*ras*, thus demonstrating that quinidine, like other HDAC inhibitors, can reverse an Ha-*ras*-induced phenotype.

Quinidine induced differentiation independently of wild-type p53. The ability of quinidine to cause differentiation of p53 mutant cell lines is consistent for a role of histone hyperacetylation in the response. HDAC inhibitors typically induce a p53-independent activation of p21^{WAF1} gene expression (5, 22). Growing MCF-7 and T47D cells express p21^{WAF1} protein in moderate to low levels (42), and quinidine raised p21^{WAF1} protein levels in MCF-7 cells approximately 11-fold within 12 h. Although p21^{WAF1} was reported to be low to undetectable in MDA-MB-231, p21^{WAF1} was detected in Western analyses of both MDA-MB-231 and T47D cells in a p53-independent fashion in response to serum deprivation, adriamycin, etoposide (42, 43), and quinidine (data not shown). These data support the idea that the p21^{WAF1} gene is present but inactive in growing MDA-MB-231 cells. Since histone hyperacetylation of the p21^{WAF1} gene occurs in response to HDAC inhibitors, it might be involved in the pathway of p53-independent activation of p21^{WAF1} gene expression (5).

The processes of cellular differentiation and cell cycle progression are interdependent. G_1 arrest is a necessary but insufficient condition for differentiation in numerous cell types including leukemic cells, keratinocytes, colonic epithelium, and muscle cells. In all of these cells, induction of p21^{WAF1} protein and G_1 cell cycle arrest occurred prior to differentiation (44–50) and was generally independent of p53. We hypothesize that the differentiated state can be viewed as a cellular response to G_1 arrest, requiring a change in gene expression profile and suppression of cell death pathways. The response of MCF-7 breast tumor cells to quinidine is consistent with this model.

To begin to understand how quinidine might elicit G_1 arrest of MCF-7 cells, we have focused on the action of quinidine as a potassium channel blocking agent. Quinidine enters cells and inhibits cardiac potassium channels by binding to the intracellular face of the ion pore (51). Although the location of the quinidine-binding site on the ATP-sensitive potassium channel is unknown, quinidine is freely permeable across membranes and inhibits the ATP-sensitive potassium channels whether it is applied to the external or internal surface of a lipid membrane bilayer (52).

In the presence of quinidine, MCF-7 cells accumulate at a position 12 h into G_1 phase (12). This position, defined by cell cycle arrest and release experiments, precedes the lovastatin arrest point by 5–6 h and is clearly distinct from the restriction

9. Xiong, Y., Zhang, H., and Beach, D. (1993) *Genes Dev.* **7,** 1572–1583
10. Sheikh, M. S., Rochefort, H., and Garcia, M. (1995) *Oncogene* **11,** 1899–1905
11. Warrell, R. P., Jr., He, L.-Z., Richon, V., Calleja, E., and Pandolfi, P. P. (1998) *J. Natl. Cancer Inst.* **90,** 1621–1625
12. Wang, S., Melkoumian, Z. K., Woodfork, K. A., Cather, C., Davidson, A. G., Wonderlin, W. F., and Strobl, J. S. (1998) *J. Cell. Physiol.* **176,** 456–464
13. Kasid, A., Lippman, M. E., Papageorge, A. G., Lowy, D. R., and Gelmann, E. P. (1985) *Science* **228,** 725–728
14. Greenspan, P., Mayer, E. P., and Fowler, S. D. (1985) *J. Cell Biol.* **100,** 965–973
15. Toscani, A., Soprano, D. R., and Soprano, K. J. (1990) *J. Biol. Chem.* **265,** 5722–5730
16. Nakajima, H., Kim, Y. B., Torano, H., Yoshida, M., and Horinouchi, S. (1998) *Exp. Cell Res.* **241,** 126–133
17. Hendzel, M. J., Delcuve, G. P., and Davie, J. R. (1991) *J. Biol. Chem.* **266,** 21936–21942
18. Sun, J.-M., Chen, H. Y., Moniwa, M., Samuel, S., and Davie, J. R. (1999) *Biochemistry* **38,** 5939–5947
19. Saunders, N., Dicker, A., Popa, C., Jones, S., and Dahler, A. (1999) *Cancer Res.* **59,** 399–404
20. Schmidt, K., Gust, R., and Jung, M. (1999) *Arch. Pharm. (Weinheim)* **332,** 353–357
21. Kim, Y. B., Lee, K. H., Sugita, K., Yoshida, M., and Horinouchi, S. (1999) *Oncogene* **18,** 2461–2470
22. Saito, A., Yamashita, T., Mariko, Y., Nosaka, Y., Tsuchiya, K., Ando, T., Suzuki, T., Tsuruo, T., and Nakanishi, O. (1999) *Proc. Natl. Acad. Sci. U. S. A.* **96,** 4592–4597
23. Sowa, Y., Orita, T., Minamikawa-Hiranabe, S., Mizuno, T., Nomura, H., and Sakai, T. (1999) *Cancer Res.* **59,** 4266–4270
24. Nakanishi, M., Kanedo, Y., Matsushime, H., and Ikeda, K. (1999) *Biochem. Biophys. Res. Commun.* **263,** 35–40
25. Levine, A. J. (1997) *Cell* **88,** 323–331
26. Ameyar, M., Shatrov, V., Bouquet, C., Capoulade C., Cai, Z., Stancou, R., Badie, C., Haddada H., and Chouaib, S. (1999) *Oncogene* **18,** 5464–5472
27. Nieves-Neira, W., and Pommier, Y. (1999) *Int. J. Cancer* **82,** 396–404
28. Stampfer, M. R., and Yaswen, P. (1993) *Cancer Surv.* **18,** 7–34
29. Stingl, J., Eaves, C. J., Kuusk, U., and Emerman, J. T. (1998) *Differentiation* **63,** 201–213
30. Jing, Y., Zhang, J., Waxman, S., and Mira-y-Lopez, R. (1996) *Differentiation* **60,** 109–117
31. Halm, H. A., Ip, M. M., Darcy, K., Black, J. D., Shea, W. K., Forczek, S., Yoshimura, M., and Oka, T. (1990) *In Vitro Cell Dev. Biol.* **26,** 803–814
32. Douglas, A. M., Grant, S. L., Goss, G. A., Clouston, D. R., Sutherland, R. L., and Begley, C. G. (1998) *Int. J. Cancer* **75,** 64–73
33. Zhang, L., Chang, C. J., Bacus, S. S., and Hung, M. C. (1995) *Cancer Res.* **55,** 3890–3896
34. Giani, C., Casalini, P., Pupa, S. M., De Vecchi, R., Ardini, E., Colnaghi, M. I., Giordano, A., and Menard, S. (1998) *Oncogene* **17,** 425–432
35. Mehta, R. R., Bratescu, L., Graves, J. M., Green, A., and Mehta, R. G. (2000) *Int. J. Oncol.* **16,** 65–73
36. Bacus, S. S., Kiguchi, K., Chin, D., King, C. R., and Huberman, E. (1990) *Mol. Carcinogen.* **3,** 350–362
37. Fuqua, S. A., Schiff, R., Parra, I., Friedrichs, W. E., Su, J. L., McKee, D. D., Slentz-Kesler, K., Moore, L. B., Willson, T. M., and Moore, J. T. (1999) *Cancer Res.* **59,** 5425–5428
38. Vladusic, E. A., Hornby, A. E., Guerra-Vladusic, F. K., Lakins, J., and Lupu, R. (2000) *Oncol. Rep.* **7,** 157–167
39. Soule, H. D., Vazquez, J., Long, A., and Albert, S. (1973) *J. Natl. Cancer Inst.* **51,** 1409–1416
40. Futamura, M., Monden, Y., Okabe, T., Fujita-Yoshigaki, J., Yokoyama, S., and Nishimura, S. (1995) *Oncogene* **10,** 1119–1123
41. Itazaki, H., Nagashima, K., Sugita, K., Yoshida, H., Kawamura, Y., Yasuda, Y., Matsumoto, K., Ishii, K., Uotani, N., Nakai, H., Terui, A., Yoshimatsu, S., Ikenishi, Y., and Nakagawa, Y. (1990) *J. Antibiot. (Tokyo)* **43,** 1524–1532
42. Sweeney, K. J., Swarbrick, A., Sutherland, R. L., and Musgrove, E. A. (1998) *Oncogene* **16,** 2865–2878
43. Sheikh, M. S., Li, X.-S., Chen, J.-C., Shao, Z.-M., Ordonez, J. V., and Fontana, J. A. (1994) *Oncogene* **9,** 3407–3415
44. Chang, B. D., Wuan, Y., Broude, E. V., Zhu, H., Schott, B., Fang, J., and Roninson, I. B. (1999) *Oncogene* **18,** 4808–4018
45. DiCunto, F., Topley, G., Calautti, E., Hsiao, J., Ong, L., Seth, P. K., and Dotto, G. P. (1998) *Science* **280,** 1069–1072
46. Evers, B. M., Ko, T. C., Li, J., and Thompson, E. A. (1996) *Am. J. Physiol.* **271,** G722–G727
47. Freemerman, A. J., Vrana, J. A., Tombes, R. M., Jiang, H., Chellappan, S. P., Fisher, P. B., and Grant, S. (1997) *Leukemia (Baltimore)* **11,** 504–513
48. Missero, C., Di Cunto, F., Kiyokawa, H., Kof, F. A., and Dotto, G. P. (1996) *Genes Dev.* **10,** 3065–3075
49. Matsumura, I., Ishikawa, J., Nakajima, K., Oritani, K., Tomiyama, Y., Miyagawa, J.-I., Kato, T., Miyazaki, H., Matsuzawa, Y., and Kanakura, Y. (1997) *Mol. Cell. Biol.* **17,** 2933–2943
50. Parker, S. B., Eichele, G., Zhang, P., Rawls, A., Sands, A. T., Bradley, A., Olson, E. N., Harper, J. W., and Elledge, S. T. (1995) *Science* **267,** 1024–1027
51. Yeola, S. W., Rich, T, C., Uebele, V. N., Tamkun, M. M., and Snyders, D. J. (1996) *Circ. Res.* **78,** 1105–1114
52. Iliev, I. G., and Marino, A. A. (1993) *Cell. & Mol Biol. Res.* **39,** 601–611
53. Pardee, A. B. (1974) *Proc. Natl. Acad. Sci. U. S. A.* **71,** 1286–1290
54. Woodfork, K. A., Wonderlin, W. F., Peterson, V. A., and Strobl, J. S. (1995) *J. Cell. Physiol.* **162,** 163–171
55. Klimatcheva, E., and Wonderlin, W. F. (1999) *J. Membr. Biol.* **171,** 35–46
56. Liu, X., Zou, H., Widlak, P., Garrard, W., and Wang, X. (1999) *J. Biol. Chem.* **274,** 13836–13840